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STUDY OF ANTIBACTERIAL ACTIVITY OF *LACTOCOCCUS LACTIS* AGAINST SPOILAGE PSYCHROTROPHIC BACTERIA ISOLATED FROM REFRIGERATED RAW MILK

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Summary

Effect of Lactococcus lactis ssp lactis inoculated in UHT skim milk on the proteolytic activity of psychrotrophic strains was studied. The titration of a purified non nitrogenous fraction from refrigerated UHT milk samples, previously inoculated by 10^6 psychrotrophic strains, showed that Pseudomonas fluorescens and P. putida were the most proteolytic species. This result has also been confirmed using polyacrylamide gel electrophoresis of casein fractions extracted from refrigerated and contaminated UHT milk samples. All extracellular proteases of the studied germs revealed a decreasing affinity towards κ, β, and α_{s1}-caseins. This bacterial proteolysis of the UHT milk caseins samples, voluntarily contaminated by each of the studied psychrotrophic strains, was significantly reduced following the addition of 10^6 CFU/ml Lactococcus, which caused, after 9 days of refrigeration, an appreciable reduction of the number of psychrotrophic germs from a minimum of 100 to 1000 times, depending on the tested strain. Our results suggest that the inhibitory effect of lactic acid bacteria on psychrotrophs is due to acidification or/and bacteriocin like production.

Key words: *Lactococcus lactis, psychrotrophic bacteria, UHT milk*

Introduction

Many microorganisms can spoil milk and dairy products [16, 19]. During milk storage by refrigeration psychrotrophs can proliferate. These bacteria hydrolyse milk proteins and lipids through the secretion of a variety of heat stable extracellular proteases and lipases, which cause different defects in dairy products [24] as curdling in UHT [11] or pasteurised milk [7], various off-flavours [9], abnormal texture and reduced cheese yields [28]. It has been shown that proteinases and lipases from psychrotrophs can be inactivated by thermal treatment between 50 °C and 65 °C. For cheese obtained directly from raw milk, this solution cannot be admitted, for this reason alternative solutions to inhibit psychrotrophs have been considered e.g. use of lactic acid bacteria (LAB); activation of the lactoperoxidase system (LPS) that needs the presence of 2 factors, hydrogen peroxide and thiocyanate to develop its antimicrobial function.

The main functions of lactic acid bacteria (LAB) are the transformation of lactose to lactic acid, flavour synthesis and direct contribution to the final texture of dairy products [22]. Diverse strains of LAB are commonly used as starters in the transformation of a variety of cheeses. These bacteria participate in the pro-

teolysis during cheese ripening especially in hydrolyzing short and medium peptides into amino acids precursors of aroma compounds, contributing to some sensorial and textural properties of cheeses [26]. Among LAB strains, different species belonging to *Lactococcus lactis* are widely used in dairy manufacture. The proteolytic system of *L. lactis* is composed of a cell-wall proteinase and ten intracellular peptidases. *L. lactis* contributes to final flavours of cheese by synthesis at the ripening stage of a wide variety of branched-chain alcohols and aldehydes resulting from branched-chain amino acids catabolism [10]. On the other hand, various studies have shown that *L. lactis* is a bacteriocin producer, specifically of Nisin that is used as a natural preservative in some foods. Also LAB are able to produce other antimicrobial substances such as lactic acid, diacetyl, carbon dioxide and hydrogen peroxide [3].

The aim of this work was to study the inhibition of spoilage gram negative psychrotroph bacteria growth by LAB. This study has been performed by inoculation of UHT skimmed milk with different stains of psychrotroph bacteria isolated from refrigerated raw milk and determination of their growth inhibition after addition of *L. lactis* ssp. *lactis* by control of casein proteolysis.

Materials and Methods

Materials. API 20 NE and API 50 CH were obtained from Biomerieux (France). PCA (Marnes-La-Coquette, France) and MRS (Beauvais, France) agar were purchased from Biorad. Nutritive broth was from Biorad (Hercules, USA). Proteins standards used in SDS-PAGE were from Biorad (France). Proteinase k (Carisbad, Germany) and Trypsin (Steinhein, Switzerland) were provided from Fluka. Trichloroacetic acid (TCA) (Darmstadt, Germany) and UHT bovine milk samples were kindly supplied by Thalja S.A. (Tunis, Tunisia).

Strains and culture conditions. Bacterial strains used in this study were isolated from refrigerated raw milk and were identified as *Aeromonas caviae* (36%), *Pseudomonas fluorescens* (29%), *P. putida* (11%), *P. cepacia* (3%) and *Chryseomonas luteola* (15%) in our laboratory [18]. They were classified using the API 20 NE. Phenotypic characteristics of these strains were treated with Statistica software version 5.0. (1997). Strains of LAB were isolated from milk and dairy products (50 strains). They were identified based on Gram staining, catalase, oxidase tests and fermentation of 49 carbohydrates (API 50 CH). Both isolates of Gram-negative germs and LAB were subcultured twice (1% inoculum, 24 h, 30 ^{o}C) in 15 ml nutritive broth (Biorad) and kept frozen at -20 ^{o}C in nutritive broth supplemented with 10 % glycerol.

Inoculation of milk. Single *P. fluorescens*, *P. putida*, *P. cepacia*, *C. luteola* or *A. caviae* was inoculated in 500 ml UHT skim milk at 10^6 CFU/ml with and without *L. lactis* ssp*. lactis* (10^6 CFU/ml) and stored at 4 ^{o}C during 9 days.

Bacterial counts. Bacterial populations were estimated by standard methods (Guiraud, 1998). Psychrotrophs and mesophilic bacteria were inoculated on PCA and incubated respecttively at 7 ^{o}C for 10 days and 30 ^{o}C during 72 h. LAB were plated on MRS and incubated 72 h at 37 ^{o}C.

Antibacterial activities. Antibacterial activity of LAB was tested against Gram-negative strains [27]. 5 µl of an overnight culture of LAB was put on MRS agar and incubated for 24 h at 30 ^{o}C. Later, 5 ml of PCA agar (0.75 % agar) inoculated with 200 µl of an overnight indicator microorganism was poured on MRS spots. The plates were then incubated for 24 h at 30 ^{o}C and were subsequently examined for zones of inhibition.

Characterization of the antibacterial compounds. LAB cultures broth samples were centrifuged (12000 g for 15 min at 4 ^{o}C) and cell-free solutions were obtained to be used in these experiments. Proteinase k and trypsin were used to conclude about the proteic nature of inhibitory factors. The enzymes were used at a final concentration of 1 mg/ml of neutralized cell free solution. Samples and controls were held at 37 ^{o}C for 1 h. Controls were prepared using sterile buffer solution instead of enzymes solutions. The samples were adjusted to pH 7 with 1N NaOH for determination of bacteriocin like stability. Heat stability of bacteriocin was measured at 80 and 100 ^{o}C for 15 s. Plates of spot on the lawn assay were prepared as described above [20].

Physico-chemical analysis. pH of UHT milk samples was measured. Proteolysis of milk caseins was detected by measuring the levels of trichloroacetic acid (TCA) soluble peptide determined by Kjeldahl method [21]. Polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 15% gels as described by Laemmli (1970) [14]. Preparation of protein samples for SDS-PAGE was conducted according to the Addeo methods [2]. Samples

were taken at regular intervals for the determination of total viable counts, psychrotrophics flora, mesophilic lactic flora, trichloroacetic acid (TCA) soluble peptide and for SDS-PAGE.

Statistical analysis. Analysis of simple linear correlation coefficients was performed using SPSS 8.0 software (Chicago, USA). Statistical significance level was defined as $P < 0.05$.

Results and Discussion

The inhibition of spoilage gram negative psychrotroph bacteria growth by LAB was studied in the present work. The majority of the psychrotrophic strains identified in raw milk were Gram-negative accounting for nearly 80 % of the total psychrotrophic microflora belonging to the two genera *Pseudomonas* (58 %) and *Aeromonas* (36 %), independently of the refrigeration storage period [18].

Antagonistic activity of each LAB of five dominant strains, isolated from raw milk was tested on gram-negative bacteria. LAB strains used were: *L. lactis* spp. *lactis* (62 %), *Lactobacillus pentosus* (6 %), *L. plantarum* (12 %), *Streptococcus thermophilus* (4 %) and *Leuconostoc* spp. *cremoris* (16 %). LAB gave circular inhibition areas onto the indicator spoilage strains tested (Fig. 1). The diameters of these inhibition areas were estimated to be between 8 mm and 15 mm. The biggest diameter of 15 mm inhibition was obtained with strain of *L. lactis spp. lactis*. However, the antibacterial property observed with *L. lactis* ssp. *lactis* has been inactivated after addition of Trypsin and Proteinase k. This result suggests the proteic nature of the antimicrobial component.

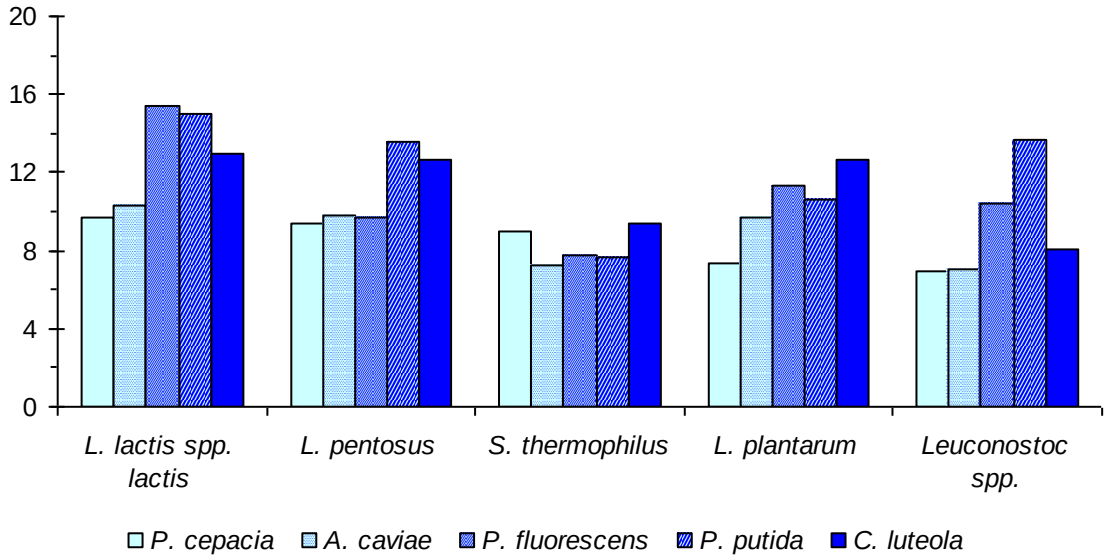

Fig. 1. Antagonistic activity of LAB on psychrotrophic bacteria.

Heating at 80 °C or 100 °C for 15 min did not induce denaturation of antibacterial compounds. Otherwise, it is interesting to note that bactericide activity was found to be stable at pH of 5 and 7 (data not shown). Stability reduction was observed by many authors [20], who reported that inhibitive substances of some LAB strains could be related with both lactic acid and proteinaceous antibacterial compounds [23].

In this study we observed that the pH of milk samples inoculated with the psychrotrophic strains (10^6 CFU/ml) undergoes a slight reduction. On the other hand, the inoculation with *Lactococcus* (10^6 CFU/ml) of raw milk induced an acidification of the medium. The pH decreease primarily takes place during the first two days of storage (1.88 pH units). Beyond this stage and until the 9^{-th} day of refrigeration, the pH of milks remained stable around an average value of 4.3 pH units independently of the tested strain (Fig. 2).

Recorded reductions are around 0.89 ($R = -0.82$), 0.51 ($R = -0.87$), 1.31 ($R = -0.98$), 0.44 ($R = -0.87$) and 0.44 ($R = -0.95$) pH units after 9 days of incubation at 4 °C of UHT milk samples, respectively supplemented with *A. caviae*, *P. fluorescens*, *P. putida*, *C. luteola* and *P. cepacia*. These results showed that the most significant reduction of pH was obtained for milk samples inoculated with strains of *P. putida*. Likewise, when milk samples are inoculated by a mixed culture, composed of a psychrotrophic

and a *L. lactis* ssp. *lactis* strains, the pH undergoes a more significant reduction. Thus, a diminution of approximately 1.88 pH units in average value is recorded after 2 days of refrigeration at 4 °C of milk independently of the psychrotrophic strain tested. During this stage and until the end of storage, the pH of the milk samples remains stable. Therefore, after 9 days of refrigeration, the pH of the different milk samples reaches the average value of 4.3 pH units. Some authors reported that the addition of 2.5×10^7 *Lactococcus*/ml to the milk reduced its pH up to the value of 6.54 after 48 h of incubation at 7 °C [8]. Under these conditions, the acidification of milk cannot be responsible for the inhibition of the psychrotrophic bacteria proliferation. On the contrary, in this case, the acidification of milk, being more important, plays a major role in the inhibition of the psychrotrophic bacteria proliferation. Moreover, the results obtained by Desmazeaud showed that the psychrotrophic bacteria cannot grow at pH ≤ 5.4 [12].

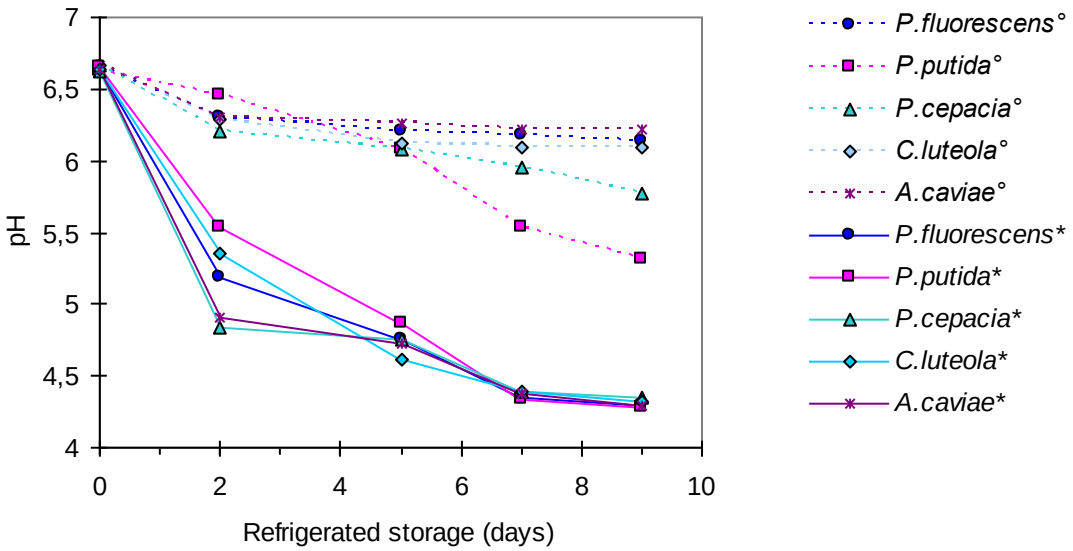

Fig. 2. pH evolution during refrigerated storage of UHT milk inoculated with psychrotrophic Gram-negative strains (10^6 CFU/ml) alone (°- - -) or with *L. lactis* ssp. *lactis* (10^6 CFU/ml) (* -—).

We observed that total mesophilic flora revealed a significant development during the refrigeration of raw milk. As it is shown in Table 1 total germs reached, after 9 days of storage at 4 oC, values of about 3×10^7, 2.5×10^{10}, 10^8, 2×10^8, 2.8×10^8 CFU/ml, respectively for milk samples inoculated by *P. fluorescens*, *P. putida*, *P. cepacia*, *C. luteola* and *A. caviae*. On the other hand, we observed that the psychrotrophic bacteria content was significantly decreased compared with their population in the UHT milk without *L. lactis.* Independently of the duration of refrigeration, the linear correlation coefficients were found equal to +0.82 (*P. fluorescens*), +0.91 (*P. putida*), +0.96 (*P. cepacia*), +0.97 (*C. luteola*) and +0.92 (*A. caviae*), against respectively +0.99, +0.93, +0.94, 0.94 and 0.90 reported for total flora as shown in Table 2. The concentration of the psychrotrophic bacteria remained high at the end of refrigeration, around an average value of 10^9 CFU/ml, whatever was the germ inoculated. At these levels, proteases and/or thermoresistant lipases production is important [1]. In addition, our results concerning the influence of the addition of lactic strain *L. lactis* spp. *lactis* on the development of each of the five psychrotrophic strains showed that total mesophilic flora continues to develop during the refrigeration of milk but at a lower rate compared to that measured in the pilot milk samples not inoculated by the lactic strain. Thus, the number of total germs reached, at the end of 9 days of refrigeration, values about 2×10^9, 2.5×10^{10}, 2×10^9, 3×10^9, 10^9 CFU/ml, respectively for samples of inoculated milk with *P. fluorescens* (R = +0.50), *P. putida* (R = +0.63), *P. cepacia* (R = +0.64), *C. luteola* (R = +0.52) and *A. caviae* (R = +0.54). Results obtained show that proliferation of psychrotrophic bacteria increased during the 2 first days of refrigeration, and varied from 3×10^6 to 1.2×10^8, from 2.5×10^6 to 9×10^7, from 1.9×10^6 to 2×10^6, from 3×10^6 to 1.1×10^8 and finally from 3×10^6 to 8.3×10^8 CFU/ml, respectively for samples of inoculated milk with *P. fluorescens*, *P. putida*, *P. cepacia*, *C. luteola* and *A. caviae*.

Table 1. Microflora development during refrigerated storage of UHT milk inoculated with psychrotrophic strains (10^6 CFU/ml) with/without *L. lactis* ssp. *lactis* (10^6 CFU/ml).

Refrigerated storage (days)	Inoculated strain	*L. lactis* ssp. *lactis*	Microflora type (CFU/ml)		
			Total flora	Lactic flora	Psychrotrophic flora
0	*P. fluorescens*	-	10^6	<10	10^6
		+	5.0×10^7	10^8	3.0×10^7
	P. putida	-	1.8×10^6	<10	2.9×10^7
		+	2.8×10^6	5.0×10^7	2.5×10^7
	P. cepacia	-	2.5×10^6	<10	2.0×10^5
		+	2.5×10^6	3.5×10^6	2.0×10^6
	C. luteola	-	3.0×10^6	<10	3.0×10^5
		+	3.0×10^7	2.8×10^7	3.0×10^6
	A. caviae	-	5.0×10^7	<10	5.0×10^7
		+	3.0×10^6	1.3×10^9	3.0×10^7
2	*P. fluorescens*	-	2.0×10^6	<10	3.0×10^8
		+	3.0×10^9	2.8×10^9	1.2×10^8
	P. putida	-	1.4×10^8	<10	3.3×10^8
		+	6.0×10^9	7.0×10^9	9.0×10^7
	P. cepacia	-	2.0×10^7	<10	5.0×10^6
		+	3.0×10^9	2.9×10^9	2.0×10^6
	C. luteola	-	3.0×10^7	<10	1.5×10^6
		+	8.0×10^9	7.1×10^9	1.1×10^8
	A. caviae	-	1.4×10^8	<10	8.0×10^7
		+	2.8×10^9	2.4×10^9	8.3×10^8
5	*P. fluorescens*	-	5.0×10^6	<10	5.0×10^8
		+	4.0×10^{10}	2.0×10^{10}	7.0×10^7
	P. putida	-	2.3×10^8	<10	2.8×10^9
		+	2.5×10^{10}	8.0×10^9	5.1×10^6
	P. cepacia	-	3.0×10^7	<10	1.2×10^7
		+	3.3×10^9	1.2×10^{10}	7.2×10^5
	C. luteola	-	5.0×10^7	<10	1.3×10^7
		+	4.0×10^{10}	7.4×10^{10}	5.0×10^7
	A. caviae	-	2.4×10^8	<10	1.0×10^8
		+	3.8×10^{10}	3.0×10^9	1.0×10^7
7	*P. fluorescens*	-	1.0×10^7	<10	7.0×10^8
		+	2.4×10^9	2.9×10^{10}	2.0×10^7
	P. putida	-	6.0×10^8	<10	3.0×10^9
		+	1.5×10^{10}	1.5×10^{10}	3.3×10^6
	P. cepacia	-	5.0×10^7	<10	1.7×10^8
		+	1.1×10^9	1.0×10^{10}	7.1×10^5
	C. luteola	-	1.7×10^8	<10	7.0×10^8
		+	4.0×10^9	2.0×10^{10}	5.7×10^6
	A. caviae	-	2.8×10^8	<10	9.5×10^7
		+	2.3×10^9	4.0×10^9	5.4×10^6
9	*P. fluorescens*	-	3.0×10^7	<10	1.2×10^9
		+	2.0×10^9	2.6×10^9	2.6×10^6
	P. putida	-	2.5×10^{10}	<10	3.1×10^9
		+	2.5×10^9	1.5×10^{10}	6.7×10^5
	P. cepacia	-	1.0×10^8	<10	2.0×10^8
		+	2.0×10^9	8.0×10^9	4.5×10^5
	C. luteola	-	2.0×10^8	<10	8.0×10^8
		+	3.0×10^9	3.0×10^{10}	8.0×10^6
	A. caviae	-	2.8×10^8	<10	2.0×10^8
		+	1.0×10^9	1.8×10^{10}	5.8×10^6

Table 2. Simple linear correlation coefficients obtained between spoilage flora inoculated, pH, non nitrogeneous fraction during storage of UHT milk at 4 °C.

Parameters	Psychrotrophic bacteria				
	P. fluorescens	*P. putida*	*P. cepacia*	*C. luteola*	*A. caviae*
Total flora $^{-}$	+0.99*	+0.93*	+0.94*	+0.94*	+0.90*
Lactic flora $^{-}$	0	0	0	0	0
Psychrotrophic flora $^{-}$	+0.82*	+0.91*	+0.96*	+0.97*	+0.92*
pH $^{-}$	-0.87*	-0.98*	-0.95*	-0.87*	-0.82*
Non proteic nitrogen $^{-}$	+0.71*	+1.00*	+0.67*	+0.06	+0.80*
Total flora $^{+}$	+0.50	+0.63*	+0.64*	+0.52**	+0.54**
Lactic flora $^{+}$	+0.65*	+0.79*	+0.77*	+0.77*	+0.91*
Psychrotrophic flora $^{+}$	-0.70*	-0.90*	-0.96*	-0.11	-0.67*
pH $^{+}$	-0.91*	-0.96*	-0.84*	-0.92*	-0.86*
Non proteic nitrogen $^{+}$	-0.17	+0.42	-0.19	0.00	-0.55**

Legend: * level of significance <1 %; ** level of significance <5 %; $^{-}$ sterilized milk inoculated with single psychrotrophic bacteria (10^6 CFU/ml); $^{+}$ sterilized milk inoculated with single psychrotrophic bacteria (10^6 CFU/ml) and with *L. lactis* ssp. *lactis* (10^6 CFU/ml).

From this stage and until the end of the storage, except for *C. luteola*, we observed that the rate of these psychrotrophic germs decreesed significantly to reach a value between 10^5 and 10^6 CFU/ml according to the tested strain. This result can be explained by an initial cohabitation (maintained for 2 days) of the lactic bacteria and the psychrotophic germs followed by a phenomenon of inhibition exerted by the *Lactococcus* bacteria on the growth of the psychrotrophic flora, which occurred mainly after the 2^{-nd} day of refrigeration. These observations showed that lactic flora and spoilage flora (*Pseudomonas* and *Aeromonas* bacteria) can coexist up to a high level of concentration. In this work, results obtained showed that LAB inhibited the proliferation of the psychrotrophic bacteria by a factor ranging between 10^2 and 10^3, depending on the inoculated psychrotrophic strain. These results are in agreement with those obtained previously by Champagne [8] who showed that addition of 2.7×10^7 *Lactococcus*/ml in sterilized milk reduces *P. putida* development of about 50 %. Moreover, these authors reported that a concentration of 2.5×10^6 of commercial *Lactococcus* per ml of milk did not reduce significantly the proliferation of psychrotrophic germs, whereas the addition of 2.5×10^7 CFU/ml decreased clearly the psychrotrophics growth 10-fold. The important decrease of the psychrotrophic bacteria content was related to the acidification of milk observed from the 2^{-nd} day of refrigeration (pH < 4.7). On the other hand, it has been shown that psychrotrophic inhibition depends also on bacteriocin produced by the *L. lactococcus* ssp. *lactis* strain as it has previously been described [6]. It has been reported that *L. lactis* was able to produce Nisin, that is the best known and most studied lanthionine-containing bacteriocin from gram positive bacteria. Nisin has inhibitory effect against some pathogens and spoilage microorganisms, including bacilli, clostridia, corynebacteria, micrococci, pediococci, staphylococci and streptococci [5].

In our study we observed that LAB undergo a significant regular increase throughout the refrigeration of milk. The initial concentrations of LAB varied from 10^8, 5×10^7, 3.5×10^6, 2.8×10^6 and 1.3×10^6 CFU/ml to reach, at the end of storage, values of 2.6×10^9, 1.5×10^{10}, 8×10^9, 3×10^{10} and 1.8×10^{10} CFU/ml, respectively for milk samples previously inoculated by *P. fluorescens* (R = +0.65), *P. putida* (R = +0.79), *P. cepacia* (R = +0.77), *C. luteola* (R = +0.77) and *A. caviae* (R = +0.91). This result suggests that enzymatic activity of psychrotrophic bacteria can favour the growth of the lactic bacteria which can use the peptides amino acids and ammonia accumulated in milk and produced by the psychrotrophic bacteria [25].

In our work, evolution of proteolysis in milk was studied. The profiles of non protein nitrogen (NPN) fractions were measured by the Kjeldhal method in various milk samples inocu-

lated by each of the five psychrotrophic bacteria strains. As it is seen in Fig. 3, results showed that during the first days of storage, the NPN fraction contents of the various UHT milk samples, inoculated with *P. fluorescens, P. cepacia and C. luteola,* underwent respective decreases of about 50.4, 45.5 and 34.7 %. This decrease could be attributed to the use of this source of NPN at the initial phase of their growth for metabolism. On the contrary, in the milk sample inoculated with *P. putida*, the NPN undergoes a relatively important increase (39.4 %), whereas this content remained almost stable in the milk sample inoculated with *A. caviae*. These observations suggest that the NPN does not constitute a limiting factor for the growth of these two strains. Beyond this stage and until the end of the refrigeration, the NPN content of the milk samples inoculated by the various psychrotrophic strains increased regularly. The total recorded increases are significant, about 276.2, 164.4, 190.7, 117.5 and 18.2 %, respectively for milk samples inoculated with *P. fluorescens* (R = +0.71), *P. putida* (R = +1), *P. cepacia* (R = +0.67), *C. luteola* (R = +0.06) and *A. caviae* (R = +0.80). These results suggest that *P. fluorescens* and *P. putida* are the most proteolytic psychrotrophic species [19]. Otherwise, the addition of *L. lactis* ssp. *lactis* (10^6 CFU/ ml) to milk samples, previously inoculated by *P. fluorescens*, *P. putida* and *P. cepacia*, caused a considerable reduction of NPN fraction during their refrigeration. The recorded decreeses are respectively of 209.3, 131.8 and 90 % in relation to samples of milk inoculated with the same psychrotrophic bacteria but in the absence of *L. lactis* ssp. *lactis*. We observed that the lactic strain did not seem to reduce the caseins proteolysis of milk samples refrigerated and inoculated previously by *C. luteola*. The relative variations observed for NPN are the same in milk samples inoculated in either the presence or absence of *L. lactis* ssp. *lactis*, respectively 110.7 and 20.1 % (Table 2). These results show that the psychrotrophic bacteria inhibition by *L. lactis* ssp. *lactis* depends on the tested strain.

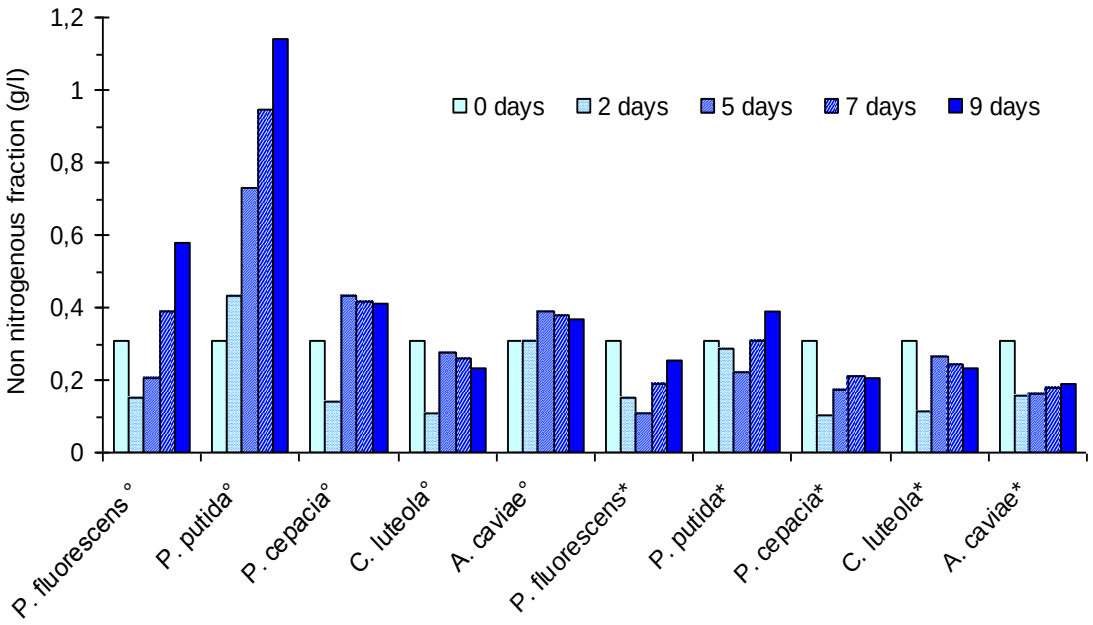

Fig. 3. Evolution of non protein nitrogen fraction during refrigerated storage of UHT milk inoculated with psychrotrophic strains (10^6 CFU/ml) and *L. lactis* ssp. *lactis* (10^6 CFU/ml).
° Sterilized milk inoculated with psychrotrophic strains ;
* Sterilized milk inoculated with psychrotrophic strains and *L. lactis* ssp. *lactis* strain.

Electrophoresis (SDS-PAGE) of casein fractions extracted from refrigerated samples of UHT milk, initially inoculated by the five studied psychrotrophs strains, used alone or in mixed culture with lactic bacteria, confirms data obtained previously on the evolution of the NPN in the time course of the milk samples refrigeration. Thus, electrophoretic profiles of caseins of the UHT milk samples, contaminated previously by every psychrotrophic strain, then refrigerated for 9 days at 4 ^{o}C (Fig. 4), present a considerable enzymatic hydrolysis of different casein fractions (α_{s1}, β and κ), accompanied with the appearance of weak molecular weights deterioration pro-

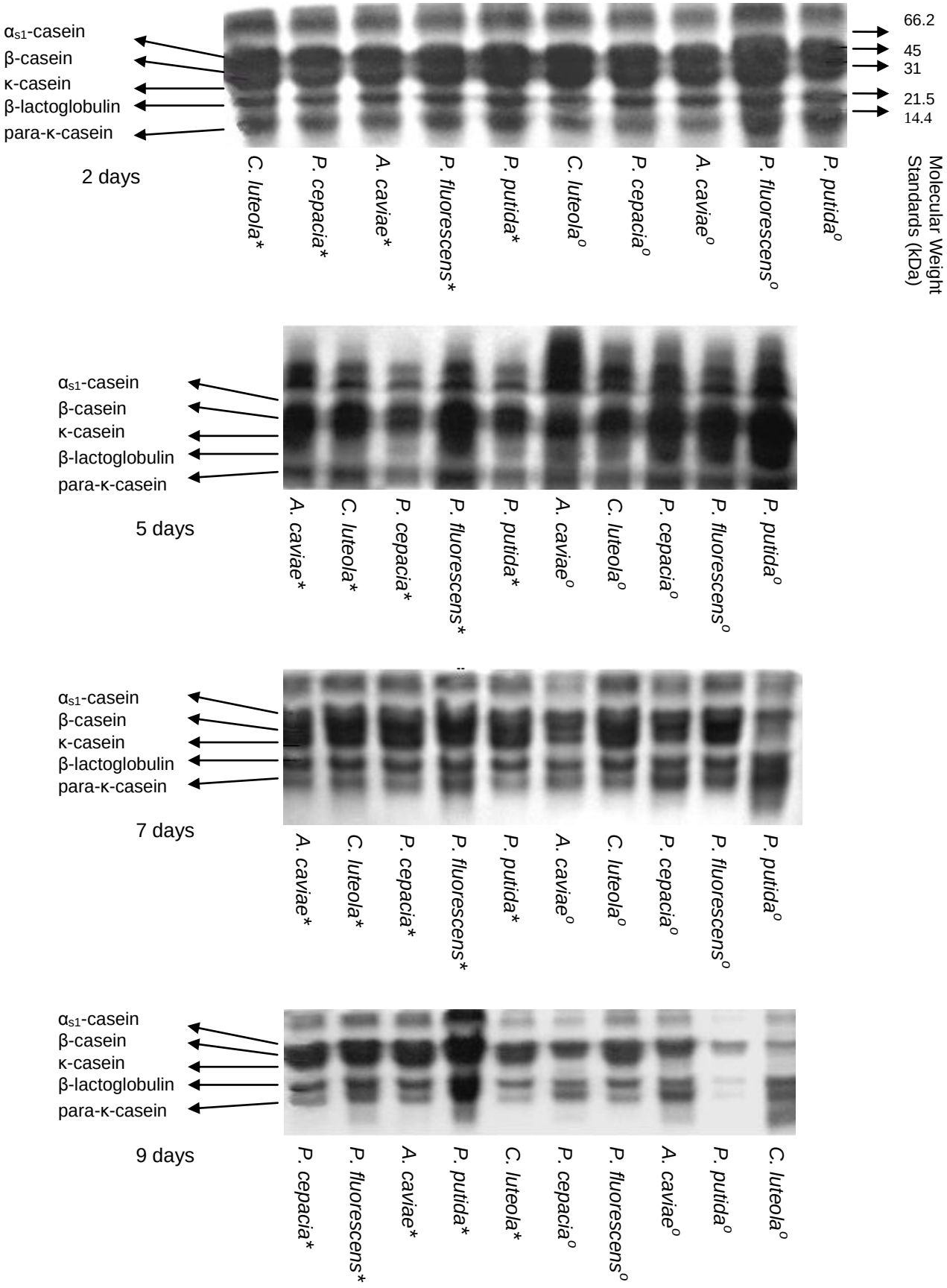

Fig. 4. Casein hydrolysis of UHT skim milk samples inoculated by psychrotrophs strains (10^6 CFU/ml), used alone (^{o}) or with *L. lactis* ssp. *lactis* (10^6 CFU/ml) (*), after 2, 5, 7 and 9 days at 4 ^{o}C was monitored by SDS-PAGE.

ducts. In the same way, we noted an increase in the strip intensity corresponding to the β-casein, because products of its deterioration have electrophoretic mobility close to that of β-casein. Otherwise, stronger deterioration of the caseins has been observed for milk samples inoculated by *P. fluorescens* and *P. putida* producing higher quantity of deterioration products, after two days of refrigeration as compared to those sowed by *P. cepacia, C. luteola* and *A. caviae*. After 9 days of refrigeration of UHT milk samples contaminated by psychrotrophic strains, these proteolytic bacteria hydrolysed β-casein faster than α_{s1}-casein and readily hydrolysed κ-casein to para-κ-casein. On the other hand, for all analyzed milk samples, the β-casein hydrolysis revealed by electrophoretic mobility showed a major band corresponding to γ-caseins. These observations are in agreement with those observed in previous studies by Law [15] who used SDS-PAGE and reported that the first phase of casein degradation by proteinase from a psychrotrophic strain, during the raw milk refrigeration, resulted in the synthesis of para-κ-casein with a concurrent decrease in the electrophorectic major band intensity of κ-casein while α_{s1}-casein was degraded at a slower rate than β-casein. The κ-casein hydrolysis by psychrtrophs' proteases was faster than that of β-casein, while the α_{s1}-casein remains less degraded. In the same way, Datte and Deeth [11] comparing the action of *Pseudomonas* proteases, showed that the chymosine attacks preferentially the κ-casein by hydrolyzing specifically only the peptide bond between Phe105 and Met106, producing hydrophobic para-κ-casein and soluble glycomacropeptide. Our results showed that in all analyzed milk samples, the addition of *L. lactis* ssp. *lactis* decreased the casein proteolysis initiated by psychrotrophic proteases Lactoserum proteins seem to be resistant to these proteases (Fig. 4). Indeed, *P. fluorescens* and *P. putida* degrade the α-lactalbumin and the β-lactoglobuline after only 9 days of the cold milk storage (Fig. 4). This result contradicts the works of Law [15] that did not observe lactoserum proteins deterioration products.

In conclusion, we showed the importance of the antagonist effect of *L. lactis* ssp *lactis* against psychrotrophic bacteria in braking proteolysis phenomena that can cause various defects: curdling in UHT [11] or pasteurized milk [7], various off-flavours [9, 16], abnormal texture, and reduced cheese yield [28]. LAB produce antimicrobial substances such as lactic acid. As perspectives we can complete this study to elucidate inhibition of initial contamination of psychrotrophic microflora gram (-) of refrigerated raw milk for cheese manufacturing by the LAB strains selected on their antagonist power.

References

1. Adams, J. B. 1991. *Int*. *J*. *Food Sci*. *Technol*., **26**, 1-20.
2. Addeo, F., M. A Nicolai, L. Chianese, L. Moio, S. Spagna Musso, A. Piccioli Bocca, L. Del Giovine, 1995. *Milchwissenschaft,* **50**, 83-85.
3. Ammor, S., G. Tauveron, E. Dufour, I. Chevallier, 2005. *Food Microbiol*., **22**, 529-539.
4. Avila, M., S. Garde, P. Gaya, M. Medina, M. Nunez, 2005. *Int. Dairy J*., **15,** 145-153.
5. Bouksaim, M., C. Lacroix, P. Audet, R. Simard, 2000. *Int. J. Food Microbiol*., **59**, 141-156.
6. Boussouel, N., F. Mathieu, A. M. Revol-Junelles, J. B. Millière, 2000. *Int. J. Food Microbiol*., **61**, 169-175.
7. Burdova, O., M. Baranova, A. Laukova, H. Rozanska, J. G. Rola, 2002. *Bull. Vet. Inst. Pulawy,* **46**, 325-329.
8. Champagne, C. P., F. Girard, M. Morin, 1990. *Rev. J. Food. Protec*., **53**, 400-403.
9. Correa, A., M. Nunes, A. Milhomem, 2000. *J. Food Microbiol*., **56**, 62-65.
10. Curtin, A. C., M. Gobbetti, P. L. H. McSweeney, 2002. *Int. J. Food Microbiol.,* **76**, 231-240.
11. Datta, N., H. C. Deeth, 2003. *Lebensm. Wiss. U. Technol*., **36**, 173-182.
12. Desmazeaud, M., 1997. *Rev. Mic. Alim. Nut.,* **15**, 99-114.
13. Guiraud, J. P., 1998. *Microbiologie alimentaire*. Dunod, Paris.
14. Laemmli, U. K. 1970. *Nature,* **227**, 680-685.
15. Law, A. J. R., J. M. Banks, D. S. Horne, J. Leaver, I. G. West, 1994. *Milchwissenschaft,* **49,** 63-67.
16. Leriche, F., A. Bordessoules, K. Fayolle, R. Kaoui, K. Laval, L. Leblanc, E. Dufour, 2004. *J. Microbiol. Methods,* **59**, 33-41.
17. Ma, Y., D. M. Barbano, M. Santos, 2003. *J. Dairy Sc. Associ.,* **86**, 1616-1631.
18. Mankaï, M., M. Hassouna, A. Boudabous, 2003. *Ind*. *Alim*. *Agric*., **120**, 12-17.
19. Mankaï, M., O. Ben Moussa, M. Hassouna, 2005. *Ind*. *Alim*. *Agric*., **122**, 7-13.
20. Meghrous, J.C., C. Barrena-Gonzalez, E. Huot, H. Petitdemange, 1995. *Microbiol. Alim. Nutri*., **13**, 255-265.
21. Moatsou, G., T. Massouras, I. Kandarakis, E. Anifantakis, 2002. *Lait,* **82**, 601-611.
22. Morales, F. J., C. Romero, S. Jimenez-Perez, 1996. *Int. J. Food Sc. Tecnol.,* **31**, 411-418.
23. Oyetayo, V.O., 2004. *African J. Biotechnol*., ***7***, 355-357.

24. Santos, J. A., T. M. Lopez-Diaz, M. C. Garcia-Fernandez, M. L. Garcia-Lopez, A. Otero, 1996. *Int. J. Food Microbiol.,* **33**, 301-306.
25. Sorhaug, T., L. Stepaniak, 1997. *Trends Food Sci. Technol*., **8**, 35-40.
26. Sousa, M. J., Y. Ardo, P. L. H. McSweeney, 2001. *Int. Dairy J.,* **11**, 327-345.
27. Villani, F., L. Sannino, G. Moschetti, G. Mauriello, O. Pepe, R. Amodio-Cocchieri, S. Copolla, 1997. *Food Microbiol.,* **14**, 555-566.
28. Zalazar, C. A., C. A. Meinardi, M. C. Candioti, M. C. 1990. *Sci. Tecn. Lattiero-Casearia,* **41**, 495-509.

ИЗСЛЕДВАНЕ НА АНТИБАКТЕРИАЛНАТА АКТИВНОСТ НА *LACTOCOCCUS LACTIS* СРЕЩУ ВРЕДНИ ПСИХРОТРОФНИ БАКТЕРИИ, ИЗОЛИРАНИ ОТ ОХЛАДЕНО СУРОВО МЛЯКО

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Резюме

Проучен е ефектът на Lactococcus lactis ssp lactis, инокулиран в UHT обезмаслено мляко (обработено при ултра висока температура), върху протеолитичната активност на психротрофни щамове. Титруването на пречистена безазотна фракция от охладени проби на UHT мляко, предварително инокулирани с 10^6 психротрофни щамове, показва, че Pseudomonas fluorescens и P. putida са видовете с най-висока протеолитична активност. Този резултат е потвърден и чрез електрофореза в полиакриламиден гел на казеинови фракции, екстрахирани от охладени и контаминирани проби на UHT мляко. Всички извънклетъчни протеази на изследваните микробни обекти показват намаляващ афинитет към κ, β, и α_{s1}-казеини. Тази бактериална протеолиза на казеиновите проби на UHT млякото, нарочно контаминирани с всеки от изследваните психротрофни щамове, се намалява значително след добавянето на 10^6 CFU/ml Lactococcus, който след 9 дни при охлаждане предизвиква осезаемо намаляване на броя на психротрофните микроорганизми не по малко от 100 до 1000 пъти, в зависимост от щама. Нашите резултати предполагат, че инхибиращият ефект на млечнокиселите бактерии върху психротрофите се дължи на подкиселяването и/или продукцията на бактериоцини.